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PRINCIPAL INVESTIGATOR: **Marcelo G. Kazanietz**

CONTRACTING ORGANIZATION: **University of Pennsylvania**
Philadelphia, PA 19104

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14. ABSTRACT

Our studies identified a novel paradigm by which PKC activation drives apoptotic responses in prostate cancer cells, which involves the autocrine release of TNFα, TRAIL, and MCP-1 (CCL2). MCP-1(CCL2) sensitizes the death effect of other cytokines through a PKC-dependent pathway. Phorbol esters control the expression and release of these cytokines/chemokines from prostate cancer cells. These effects are primarily mediated by PKCα and PKCδ. On the other hand, PKCε opposes these effects, and confer protection for apoptosis. Our studies also identified an important role for androgens in the control of the secretion of cytokines/chemokines from prostate cancer cells. In summary,

15. SUBJECT TERMS
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INTRODUCTION

The genesis and progression of prostate cancer involves a series of both genetic and epigenetic changes that lead to deregulation of mitogenic and survival signals. A key signaling element in growth factor and cytokine pathways is protein kinase C (PKC), a family of 10 related serine-threonine kinases. This family comprises the classical (cPKCs α, β, and γ), novel (nPKCs δ, ε, η, and θ), and atypical (aPKCs ζ and λ). Only cPKCs and nPKCs are regulated by phorbol esters and diacylglycerol (DAG), a lipid second messenger generated upon activation of G-protein-coupled receptors and tyrosine-kinases (1, 2).

At the cellular level, phorbol esters are capable of promoting opposite responses (mitogenesis/survival *vs.* growth arrest/apoptosis). This paradigm of functional diversity is exemplified by the novel PKCs: whereas in most cases PKCε acts as a mitogenic or anti-apoptotic kinase, PKCδ generally inhibits proliferation or triggers an apoptotic response. PKCε can signal to mitogenesis via Raf/MEK/ERK and cyclin D1 induction or even act as an oncogene (1). Interestingly, androgen-dependent prostate cancer cells undergo apoptosis in response to phorbol esters (3-5).

Work from our laboratory and others established PKCδ as the major mediator of the apoptotic effect of phorbol 12-myristate 13-acetate (PMA) in LNCaP cells. Indeed, phorbol ester-induced apoptosis can be abolished by pre-treatment with PKCδ inhibitors or by PKCδ RNAi depletion (4-6). PKCε, a phorbol ester-responsive nPKC expressed in LNCaP cells seems to have a pro-survival role in prostate cancer cells (7). Signaling analysis revealed key roles for p38 and JNK cascades in PMA-induced apoptosis (6, 8). We have also demonstrated that PKCδ-mediated prostate cancer death involves the activation of an apoptotic autocrine loop that triggers the activation of the extrinsic apoptotic cascade. Conditioned medium (CM) collected from LNCaP cells treated with PMA (CM-PMA) has pro-apoptotic activity when added to another culture of LNCaP cells, while CM collected from vehicle-treated cells (CM-Veh) had no effect on cell viability (8).

Subsequent studies established that the autocrine death pathway implicated the release of cytokines, primarily TNFα, and also TRAIL and CCL2 (MCP-1). A main objective of the DOD grant involved the characterization of the autocrine death pathway in the context of PKC signaling. Key goals were the characterization of the secreted factors, the regulation of the secretion by PKC isozymes and hormones (androgens), and establishing the relationship between individual PKC isozymes and cytokines as mediators of phorbol ester-induced apoptosis in prostate cancer cells.

Note: although in our grant application we used the name MCP-1, this cytokine will be referred as CCL2 in accordance with the current nomenclature.

BODY

A summary of the main findings is presented below. Relevant figures for points #1 through #5 have been presented in Annual Reports for years 2008 and 2009. Parts of these studies have been published, submitted for publication, or represent the core of 2 manuscripts that are now in preparation.

1. PMA stimulates the expression and secretion of CCL2 from LNCaP cells through PKC activation: role of individual PKCs. We found that PMA caused a marked release of CCL2 from LNCaP cells, as determined by ELISA. CCL2 secretion was blocked by the pan-PKC inhibitor GF109203X (bisindolilmaleimide I), arguing that CCL2 release is mediated by PKC isozymes. We also found using Q-PCR a significant induction of CCL2 mRNA levels after PMA treatment in LNCaP, DU-145 and PC-3 cells.

Next, we determined the role of specific PKC isozymes using pharmacological inhibitors and RNAi. Both the cPKC inhibitor Gö6976 (PKCα-selective in these cells, as PKCα is the only cPKC present in LNCaP cells) and the PKCδ inhibitor rottlerin dose-dependently inhibited CCL2 secretion induced by PMA. In addition, depletion of these two PKCs from LNCaP using RNAi reduced CCL2 release in response to PMA.

2. Microarray studies validated the effect of PMA on CCL2 mRNA induction and identified novel potential mediators of the PMA effect. When LNCaP cells were pre-treated with the protein synthesis inhibitor cycloheximide, the apoptotic response of PMA was reduced by 44% ($p<0.05$), suggesting the involvement of newly synthesized proteins and a potential transcriptional component. As phorbol esters are strong inducers of gene expression, we analyzed changes in gene expression using Affymetrix microarrays. These studies were carried out at the UPenn Microarray Facility. We studied the dynamics of changes in gene expression by PMA at different times between 0 and 24 h after PMA treatment. We identified ~ 5,000 PMA-genes up- or down-regulated by PMA (> 2-fold change), identified early and late genes, and classified them based on gene ontology analysis. Of particular interest for the work funded by DOD, PMA induces a strong up-regulation of cytokines. Remarkably, we found CCL2 as one of the highest PMA-up-regulated genes (> 1,000-fold change), which validates our results using Q-PCR. Other cytokines and chemokines, including IL-8, CCL8, CLC20, CXCL10, CXCL11, and Fas, as well as receptors such as CXCR4, TNFRSF11, TNFRSF12, and IL6R, are prominently up-regulated.

We carried out the first PKC isozyme-specific microarray analysis. For this study we treated with PMA (100 nM, 4 h) LNCaP cells subject to RNAi depletion for PKCα, δ, or ε (2 different duplexes/PKC). We identified multiple genes that are regulated specifically by each PKC. PKCδ was the most prominent "selective" PKC implicated gene expression, with more than 100 genes up-regulated exclusively by this nPKC. Changes in gene expression were validated by Q-PCR. We identified two novel pro-apoptotic genes that we will study beyond the scope of the current grant.

3. CCL2 release is mediated by p38. We found that a p38 inhibitor (SB203580) blocks the release of CCL2 from LNCaP prostate cancer cells. We analyzed the role of p38 on the secretion of CCL2 from LNCaP cells. The p38 inhibitor SB203580 caused a significant inhibition of CCL2 secretion. This result points to a role for p38 MAPK in CCL2 secretion in response to PKC activation.

4. CM-PMA activates p38 MAPK and induces Akt dephosphorylation. LNCaP cells have elevated Akt activity due to PTEN inactivation. We have previously determined that PMA causes a rapid dephosphorylation and inactivation of Akt in LNCaP cells. We also found that CM-PMA causes a fast dephosphorylation of Akt and a significant activation of p38 MAPK. We

speculated that the release of TNFα, TRAIL, and CCL2 lead to apoptosis by activation of the pro-apoptotic p38 MAPK pathway and by inhibition of the pro-survival Akt pathway.

5. CCL2 is involved in the apoptotic effect of PMA. Neutralizing antibodies against TNFα and/or TRAIL reduce the apoptotic effect of the phorbol ester. Interestingly, we found that addition of a CCL2 blocking antibody significantly enhanced this effect, suggesting that TNFα, TRAIL and CCL2 secreted to the medium in response to PMA contribute to the pro-apoptotic effect of CM-PMA. A thorough analysis using pharmacological inhibitors and RNAi for individual PKC isozymes established that both PKCα and PKCδ mediate the sensitizing effect of CCL2. Our data strongly suggest that CCL2 secreted to the CM contributes to the apoptotic effect of the phorbol ester in LNCaP cells.

6. The release of apoptotic factors from LNCaP cells is subject to androgen control (Mol. Carcinogenesis 48:187-195, 2009; paper attached). Our previous studies determined that PKCδ expression is transcriptionally regulated by androgens (9). As PKCδ plays a major role in the release of cytokines as well as an effector of the cytokine death effects (8), we speculated that androgen-depletion should impair the secretion of autocrine factors from LNCaP cells. Indeed, we found that the PKCδ-dependent autocrine mechanism is greatly influenced by androgens. Androgen depletion, which down-regulates PKCδ expression, greatly diminishes TNFα and TRAIL mRNA induction and release by PMA, which results in a reduced apoptogenic activity of the CM and an impaired ability of the CM to activate p38 MAPK and JNK. These effects can be rescued by addition of the synthetic androgen R1881. Our data is supported by studies using androgen receptor RNAi depletion. These results are very important because they suggest that PKC-mediated induction of death factor secretion and apoptosis in LNCaP prostate cancer cells are highly sensitive to hormonal control.

7. PKCε has a pro-survival role in LNCaP cells through the modulation of death factor secretion and as a downstream effector: opposite roles for PKCδ and PKCε (submitted for publication). There is extensive literature indicating that novel PKCs exert opposite cellular effects. Indeed, PKCδ and PKCε isozymes have generally anti-growth/apoptotic and mitogenic/pro-survival roles in many cellular models, respectively, including in prostate cancer cells. In the context of prostate cancer this is very important because the balance in PKC isozyme expression is markedly altered in human prostate tumors, potentially reflecting their involvement in the etiology and progression of the disease. Specifically, PKCε expression is up-regulated in prostate cancer specimens (10, 11). We wished to determine whether PKCδ and PKCε have different effects on the modulation of the secretion of death factors from prostate cancer cells.

The major findings were as follows:

a. PKCε RNAi depletion or expression of a PKCε-dominant-negative mutant by adenoviral means potentiates PMA-induced apoptosis in LNCaP cells. Moreover, PKCε RNAi depletion sensitizes DU145 cells to PMA-induced apoptosis even if DU145 cells do not normally undergo apoptosis in response to phorbol esters.
b. Overexpression of PKCε in LNCaP cells using an adenovirus protects LNCaP cells against apoptosis induced by PMA. This effect is proportional to the expression levels. Thus, unlike PKCδ, which is a pro-apoptotic kinase, PKCε drives survival responses in LNCaP cells.

c. PKCε modulates PMA-induced secretion of TNFα from LNCaP cells. To assess a potential implication of PKCε in this autocrine response, we collected CM from LNCaP cells subject to PKCε RNAi depletion or from control cells, either treated with PMA (CM-PMA) or vehicle (CM-Veh). CM-PMA collected from PKCε-depleted cells showed a higher apoptogenic activity when added to naïve cultures of LNCaP cells As expected, CM-Veh did not induce apoptosis in LNCaP cells. As TNFα is the primary death factor implicated in PMA-induced apoptosis in LNCaP cells, we next determined TNFα levels by ELISA. Notably, while PKCε RNAi depletion did not modify basal TNFα levels, TNFα release by PMA stimulation was markedly higher in PKCε-depleted cells relative to control cells.
d. PKCε depletion modulates the apoptotic response of death factors. LNCaP cells subject to either PKCε RNAi or control RNAi were treated with CM-PMA. Interestingly, the apoptotic rate in response to CM-PMA was higher in PKCε-depleted cells relative to control cells. Moreover, PKCε-depleted LNCaP cells were more sensitive to TNFα-induced apoptosis than control cells. Altogether, these results indicate that PKCε not only negatively modulates the secretion of pro-apoptotic factors from LNCaP cells, but that this kinase is also implicated as an effector of the death factor response.
e. PKCε inhibits JNK activation by TNFα. We found that activation of JNK by TNFα, as determined by Western blot using a phospho-JNK antibody, was higher in PKCε-depleted cells than in control LNCaP cells. On the other hand, no differences in phospho-p38 levels in response to TNFα were found between PKCε-depleted and control LNCaP cells. On the other hand, activation of JNK by TNFα in PKCε-overexpressing cells was significantly reduced. These results suggest that PKCε negatively regulates the activation of JNK in response to TNFα in LNCaP cells.
f. Additional mechanistic studies that are beyond the scope of the present grant identified the pro-apoptotic protein Bad as a key mediator of the PKCε pro-survival effect in LNCaP cells, and that PKCε translocates to the mitochondria in response to PMA.

KEY RESEARCH ACOMPLISHMENTS

- We determined that CCL2 is released and its mRNA induced in response to PMA treatment in multiple prostate cancer cells.
- Using isozyme-specific PKC inhibitors and RNAi, we found that the PMA response (both CCL2 mRNA induction and release) is sensitive to PKCα and PKCδ inhibition/depletion.
- We established an essential role for the p38 MAPK pathway in the release of CCL2 from LNCaP cells induced by PMA.
- We found that CM-PMA, when added to LNCaP cells, induces Akt dephosphorylation, suggesting that the released factors inhibit survival pathways.
- Using anti-TNFα, anti-TRAIL, and anti-CCL2 antibodies we determined that these cytokines/chemokines mediate phorbol ester-induced apoptosis in LNCaP cells.
- We established a key role for PKCα and PKCδ as mediators of the sensitizing effect of CCL2.
- We identified an important role for androgens in the control of the release of autocrine death factors from prostate cancer cells.

- Androgen depletion or androgen receptor RNAi impaired the activation of p38 MAPK and JNK pathways in response to PMA. Androgens are therefore key modulators of cytokine release and apoptosis in response to PKC activation in prostate cancer cells.
- We determined that PKCδ and PKCε have opposite roles, as PKCε is a pro-survival kinase in LNCaP cells.
- PKCε modulates PMA-induced secretion of TNFα from LNCaP cells. TNFα release by PMA stimulation was markedly higher in PKCε-depleted cells relative to control cells.
- PKCε depletion modulates the apoptotic response of death factors. Thus, PKCε not only negatively modulates the secretion of pro-apoptotic factors from LNCaP cells, but it is also implicated as an effector of the death factor response. This PKC opposes PKCδ for both responses.
- PKCε inhibits JNK activation by TNFα in LNCaP cells.

REPORTABLE OUTCOMES

Xiao, L., Caino, M.C., von Burstin, V.A., Oliva, J.L., Kazanietz, M.G. Phorbol ester-induced apoptosis and senescence in cancer cell models. Methods Enzymol. 446:123-139 (2008).

Xiao, L., Gonzalez-Guerrico, A., Kazanietz, M.G. PKC-mediated secretion of death factors in LNCaP prostate cancer cells is regulated by androgens. Mol. Carcinogenesis 48:187-195 (2009).

Meshki, J., Caino, M.C., von Burstin, V.A., Griner, E., Kazanietz, M.G. Regulation of prostate cancer cell survival by protein kinase C ε (PKCε) involves Bad phosphorylation and modulation of the TNFα/JNK pathway. Submitted for publication (2010).

CONCLUSIONS

Our studies established a novel paradigm in PKC-driven apoptosis in prostate cancer cells. Phorbol ester-induced apoptosis in LNCaP cells is mediated by the release of autocrine factors that include TNFα, TRAIL, and CCL2. Phorbol esters control the expression and release of these cytokines/chemokines, and in that regard PKCα and PKCδ are major players. We also identified a key role for PKCε in LNCaP cell survival through the modulation of the secretion and actions of death factors. Lastly, our studies identified an important role for androgens in the control of apoptosis induced by PKC activation in LNCaP cells. Androgens modulate the secretion of cytokines/chemokines from LNCaP cells. Thus, this pathway is controlled at multiple levels.

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APPENDIX

2 Papers.

CHAPTER SEVEN

Phorbol Ester–Induced Apoptosis and Senescence in Cancer Cell Models

Liqing Xiao,[*,1] M. Cecilia Caino,[*,1] Vivian A. von Burstin,[*,1] Jose L. Oliva,[†] *and* Marcelo G. Kazanietz[*]

Contents

Abstract

Protein kinase C (PKC) isozymes catalyze the phosphorylation of substrates that play key roles in the control in proliferation, differentiation, and survival. Treatment of cells with phorbol esters, activators of classical and novel PKC isozymes, leads to a plethora of responses in a strict cell-type–dependent specific manner. Interestingly, a few cell models undergo apoptosis in response to phorbol ester

* Department of Pharmacology and Institute for Translational Medicine and Therapeutics (ITMAT), University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania
† Unidad de Biología Celular, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Carretera Majadahonda-Pozuelo, Madrid, Spain
[1] These three authors contributed equally

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stimulation, including androgen-dependent prostate cancer cells. This effect involves the autocrine secretion of death factors and activation of the extrinsic apoptotic cascade. We have recently found that in other models, such as lung cancer cells, phorbol esters lead to irreversible growth arrest and senescence. This chapter describes the methods we use to assess these phorbol ester responses in cancer cell models, focusing on apoptosis and senescence.

1. Introduction

Despite early studies suggesting a role for protein kinase C (PKC) serine/threonine kinases in mitogenesis and transformation, it became clear that individual PKC isozymes can also have important roles either as modulators of pro-apoptotic or growth inhibitory responses (Black, 2000; Detjen *et al.*, 2000, Frey *et al.*, 1997, Gavrielides *et al.*, 2004; Oster *et al.*, 2006). Studies with phorbol ester tumor promoters have unambiguously implicated PKC as a family of tumor-promoting kinases, because PKC activation promotes the clonal expansion of an initiated cell. Many years have passed since these initial observations, and the field has gone a long way to establish that the paradigm is not as simple as initially thought, primarily because individual members of the PKC family play distinct (or sometimes overlapping) roles in mitogenesis, differentiation, survival, and cell death (Dempsey *et al.*, 2000; Jaken *et al.*, 2000; Mischak *et al.*, 1993; Ron *et al.*, 1999; Schechtman *et al.*, 2001; Weinstein *et al.*, 1997). Because phorbol esters target classical (calcium-dependent) PKCs α, βI, βII, and γ, as well as novel (calcium-independent) PKCs δ, ε, η, and θ, responses in most cases will depend on the distinctive expression pattern of PKC isozymes in a particular cell type, as well as to the differential expression of PKC targets and downstream effectors, which unfortunately have not yet been fully defined. PKCs differ not only in their biochemical properties but also in tissue distribution and intracellular localization, and they are subject to translocation to membrane compartments upon stimulation with either phorbol esters or ligands that activate plasma membrane receptors that generate diacylglycerol (DAG). Responses to phorbol esters can also be mediated by proteins unrelated to PKC isozymes, including protein kinase D (PKD) isozymes, chimaerins, and Ras GRPs (Colon-Gonzalez *et al.*, 2006).

2. PKCδ as a Pro-Apoptotic Kinase

Studies in hematopoietic cells have recognized that activation of PKC can signal toward apoptosis. In 1995, Emoto *et al.* demonstrated that in U-937 cells, ionizing radiation proteolytically cleaves the novel PKCδ isozyme to generate a ~40 kDa constitutively active fragment (Emoto

et al., 1995). Cleavage of PKCδ was found to occur adjacent to an Asp residue at a site with homology to that involved in proteolytic activation of interleukin-1-beta-converting enzyme (ICE). Subsequent studies demonstrated that overexpression of the catalytic fragment of PKCδ in cells is associated with an apoptotic response, as revealed by characteristic chromatin condensation, nuclear fragmentation, and accumulation of cells in sub G0/G1 phase (Bharti *et al.*, 1998; Denning *et al.*, 1998; 2002, Ghayur *et al.*, 1996, Sitailo *et al.*, 2004). A similar link between the generation of a PKCδ catalytic fragment and apoptosis was later demonstrated in several other cell types, including keratinocytes, neurons, and salivary gland cells. PKCδ cleavage occurs in response to various stimuli, such as DNA damage, UV radiation, and death factors (tumor necrosis factor-alpha or TNFα) (Basu *et al.*, 2001; Denning *et al.*, 1998; 2002; DeVries *et al.*, 2002). Cleavage was found to be dependent on caspase-3, as caspase inhibitors prevented PKCδ catalytic fragment formation as blocked the apoptotic response (Reyland *et al.*, 1999; Sitailo *et al.*, 2004). Studies from Reyland and coworkers determined that nuclear translocation of the PKCδ catalytic fragment is highly relevant for apoptosis, and a nuclear localization signal in the C-terminal region of PKCδ has been identified as responsible for nuclear shuttling in response to etoposide. PKCδ mutants lacking a functional nuclear localization signal are, indeed, unable to trigger an apoptotic response (DeVries *et al.*, 2002). In some models, mitochondrial localization of PKCδ is required for its apoptotic activity (Denning *et al.*, 2002).

It also became evident that tyrosine phosphorylation of specific residues in PKCδ plays a modulatory role in apoptosis induced by DNA damaging agents, as initially described by Brodie, Blumberg and coworkers (Blass *et al.*, 2002). The PKCδ caspase cleavage site located in the hinge region that connects the regulatory and kinase domains is flanked by tyrosines 311 and 332. A recent study in glioma cells has determined that phosphorylation of tyrosine 311 in PKCδ by c-Abl, but not Src, Lyn, or Yes, contributed to the apoptotic effect of H_2O_2, and a phosphomimetic PKCδ mutation in that position (Y→E) induced glioma cell apoptosis through the p38 MAPK pathway. Tyrosine phosphorylation in position 332 is enhanced by TRAIL and cisplatin and modulates the cleavage of the catalytic domain, as well as apoptotic function, suggesting a key regulatory role for tyrosine phosphorylation in the hinge region (Brodie *et al.*, 2003).

3. PKCδ as a Mediator of Apoptotic Responses in Prostate Cancer Cells: The Emergence of an Autocrine Paradigm

One of the first models in which phorbol esters were found to trigger an apoptotic response was the androgen-dependent LNCaP prostate cancer cell line. Early studies by Powell and coworkers, as well as several others,

have established that treatment of LNCaP cells with phorbol 12-myristate (PMA or TPA) leads to apoptotic cell death (Powell *et al.*, 1996). A typical experiment is shown in Fig. 7.1(left panel). LNCaP apoptosis is preceded by $p21^{cip1}$ up-regulation and Rb dephosphorylation (Blagosklonny *et al.*, 1997). Moreover, $p21^{cip1}$ depletion with siRNA markedly impaired the apoptotic response of PMA in these cells (Xiao and Kazanietz, unpublished observations). Surprisingly, only androgen-dependent cells undergo apoptosis in response to phorbol esters.

Our laboratory has extensively studied the molecular basis of the apoptotic effect of phorbol esters in prostate cancer cells. Adenoviral delivery of PKCδ markedly potentiates the apoptotic response of PMA in LNCaP cells. On the other hand, expression of a dominant-negative (kinase-deficient) PKCδ mutant in LNCaP cells reduced the phorbol ester response (Fujii *et al.*, 2000). Similarly, transient PKCδ depletion with siRNA or stable expression of an shRNA against this novel PKC impaired PMA-induced apoptosis (Gonzalez-Guerrico *et al.*, 2005). Notably, we found that induction of LNCaP cell death by PMA does not depend on PKCδ proteolytic cleavage by caspase-3. PMA causes a marked translocation of PKCδ to the plasma and nuclear membranes, although the site of action of this PKC in LNCaP cells is still a matter of debate. Most probably, kinase activation depends on an allosteric mechanism rather than proteolytic activation (Fujii *et al.*, 2000).

Recent studies from our laboratory have determined a key role for the extrinsic apoptotic cascade in phorbol ester–induced apoptosis in LNCaP cells. Conditioned medium (CM) collected from PMA-treated cells (CM-PMA) has apoptogenic activity when added to an untreated LNCaP culture Fig. 7.1(right panel). CM collected from androgen-independent cell lines DU-145 and PC3 cells treated with PMA also triggered cell death when

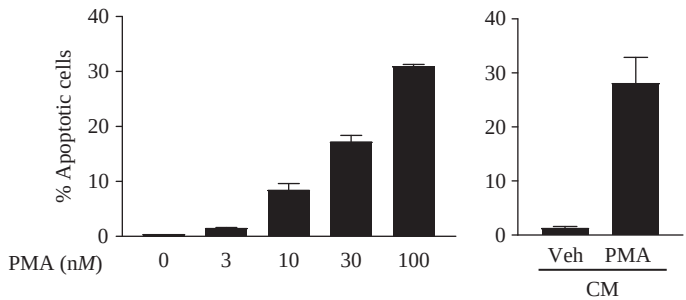

Figure 7.1 Apoptosis induction by PMA in LNCaP prostate cancer cells involves the secretion of autocrine factors. *Left panel*, LNCaP cells were treated with different concentrations of PMA (0-100 n*M*) for 1 h, and apoptosis was assessed 24 h later by DAPI staining. *Right panel*, LNCaP cells were incubated for 24 h with conditioned medium (CM) collected from LNCaP cultures treated either with vehicle (*Veh*) or PMA (100 n*M*, 1 h). Apoptotic cells were scored by DAPI staining.

added to LNCaP cells. On the other hand, CM-PMA from NIH-3T3 cells lacks apoptogenic activity. Repetitive washings after addition of the phorbol ester, which prevents the accumulation of apoptotic factors, abolishes the apoptotic response, suggesting that factors released to the medium are essential in conferring cell death. It seems that PKCδ plays a significant role in death factor release, because PKCδ inhibition or depletion by RNAi impairs the apoptotic activity of the CM-PMA (Gonzalez-Guerrico *et al.*, 2005).

An extensive analysis that used cytokine-neutralizing antibodies, death-receptor blocking antibodies, and RNAi for death receptors revealed that TNFα and TRAIL play a significant role in this response. However, the limited ability of these two cytokines to induce apoptosis in LNCaP cells is suggestive of additional factors being released to the CM in response to phorbol esters that sensitize cells to the death factors. The involvement of TNFα was further demonstrated by the ability of TAPI-2, an inhibitor of the metalloproteinase TACE/ADAM17 (TNFα-converting enzyme), and TAPI-2 RNAi, to reduce the apoptotic activity of the CM from PMA-treated LNCaP cells. PMA also causes a significant up-regulation of TNFα mRNA levels and promotes the secretion of this cytokine to the medium. TNFα secretion is markedly reduced on PKCδ RNAi (Gonzalez-Guerrico *et al.*, 2005).

Further experimental support for the involvement of the extrinsic apoptotic cascade in PMA-induced apoptosis comes from signaling studies. It is well established that stimulation of death receptors by TNFα and TRAIL activates a number of signaling cascades, including the JNK, p38 MAPK, and NF-κB pathways (Deng *et al.*, 2003; Lin *et al.*, 2000; 2004; Luschen *et al.*, 2004; Takada *et al.*, 2004; Weldon *et al.*, 2004). Emerging evidence argues for the involvement of p38 MAPK and JNK in prostate cancer cell apoptosis (Park *et al.*, 2003; Shimada *et al.*, 2003; Tanaka *et al.*, 2003). Our studies found that CM-PMA causes a significant activation of JNK and p38 MAPK in LNCaP cells, and in addition it promotes caspase-8 cleavage. Pharmacologic inhibition of p38 MAPK and JNK significantly blocked the apoptotic effect of the CM-PMA but did not affect the release of apoptotic factors by PMA, suggesting that these pathways are primarily involved as effectors of the released death factors. Furthermore, RNAi depletion of caspase-8 or the adaptor FADD impairs the ability of the CM-PMA to promote apoptosis (Gonzalez-Guerrico *et al.*, 2005). Altogether, these studies argue for the contribution of the extrinsic apoptotic cascade in the autocrine effect triggered by PKC activation in LNCaP cells.

4. Roles of PKCα and PKCε in LNCaP Cell Death and Survival

LNCaP cells do not only express PKCδ but also the classical PKCα, the novel PKCε, and the atypical PKCζ (Fujii *et al.*, 2000). Early studies by Powell and coworkers have established a correlation between membrane-associated

PKCα and apoptosis (Powell *et al.*, 1996). The diacylglycerol analog HK654, which competes with phorbol esters for binding to PKCs, triggers an apoptotic response in LNCaP cells by specifically activating PKCα. This analog causes a differential relocalization of PKCs to intracellular compartments, and its ability to cause apoptosis correlates with its ability to target PKCα to the plasma membrane. Consistently, PKCα overexpression potentiates PMA-induced apoptosis and, conversely, a dominant-negative (kinase-deficient) PKCα mutant inhibits PMA-induced apoptosis (Garcia-Bermejo *et al.*, 2002). Signaling studies revealed that this classical PKC is responsible for causing Akt dephosphorylation in LNCaP cells, probably by sensitizing cells to death factors. Because cell death can be rescued by an active Akt1 mutant (Myr-Akt1), it is believed that Akt dephosphorylation is an obligatory event for the apoptotic effect of phorbol esters (Tanaka *et al.*, 2003).

The role of PKCε in LNCaP cells is less clear, but this PKC probably mediates survival signaling, as shown in several other cellular models (Ding *et al.*, 2002; Gillespie *et al.*, 2005; Lu *et al.*, 2006; Okhrimenko *et al.*, 2005; Pardo *et al.*, 2006). Terrian's laboratory demonstrated that PKCε overexpression enhances mitogenesis through ERK and favors the transition of LNCaP cells to an androgen-independent state (Wu *et al.*, 2002a; 2002b). Unpublished studies from our laboratory also suggest that PKCε has a pro-survival role in LNCaP cells, because PKCε siRNA significantly potentiates the apoptotic effect of PMA in these cells (Meshki, J., M. C. C., and M. G. K., unpublished observations).

5. Regulation of Cell Cycle and Senescence by PKC

Cellular senescence describes the permanent withdrawal from the cell cycle in response to diverse stresses, such as dysfunctional telomeres, DNA damage, strong mitogenic signals, or disrupted chromatin (Campisi, 2005). Increasing evidence indicates that cellular senescence is a critical effector program in response to DNA-damaging chemotherapeutic agents. Despite promising results of early-life survival in cancer patients, which justifies the therapeutic application of premature senescence as a tumor suppressor mechanism, senescent cells can contribute to aging and age-related diseases in renewable tissues can contribute to aging and age-related diseases (Campisi, 2005; Schmitt, 2007). Dimri and colleagues introduced a specific senescence-associated-β-galactosidase (SA-β-Gal) marker (Dimri *et al.*, 1995), which is currently used as a biomarker of senescence. There is consensus that this method can be used to distinguish between senescent cells and quiescent cells in heterogeneous cell populations and aging tissues, such as skin biopsies from older individuals (Itahana *et al.*, 2007).

PKC activation by phorbol esters has been associated with cell cycle arrest, either in G1 or G2, and it can cause a marked change in the expression of cyclins and Cdk inhibitors such as $p21^{cip1}$, as well as in the activity of various Cdks (Table 7.1). Recently, two independent laboratories reported the involvement of PKC in senescence of human fibroblasts and melanoma cells (Cozzi *et al.*, 2006; Takahashi *et al.*, 2006). In our laboratory, we have found that phorbol ester treatment induces a senescence-like phenotype in lung cancer and colon cancer cell lines, which is characterized by morphologic changes (cell enlargement, flattening of cells, increased vacuolization) (Fig. 7.2A), irreversible cell cycle arrest, inhibition of DNA synthesis, and the expression of senescence-specific markers (Campisi, 2005). Non-small cell lung carcinoma (NSCLC) cells treated with PMA display senescence-like morphologic changes as soon as 1 day after treatment. SA-β-Gal activity is increased approximately the third day after phorbol ester stimulation (Fig. 7.2B). By use of RNAi and pharmacological approaches we established that the phorbol ester effect is mediated by the classical PKCα, and it involves the up-regulation of $p21^{cip1}$ at the transcriptional level (Oliva *et al.*, 2008).

6. Determination of Phorbol Ester–Induced Apoptosis in Prostate Cancer Cells

For these assays we use LNCaP human prostate cancer cells and media from the American Type Culture Collection (ATCC; Manassas, VA). Cells are cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT) and penicillin (100 U/ml)-streptomycin (100 μg/ml) (Gibco, Grand Island, NY) at 37deg;C in a humidified 5% CO_2 atmosphere. We normally use low passage cells (<8 passages), as growth properties change with subsequent passages and cells start becoming androgen-independent and resistant to apoptosis. We have noticed marked differences in LNCaP growth properties depending on the serum. Therefore,

Table 7.1. Cell cycle distributions 24 h after treatment with PMA (does not include sub-G0/G1)

Cell line	G0/G1 (%)	S (%)	G2/M (%)
H358 (lung cancer)	Veh: 49	Veh: 18	Veh: 21
	PMA: 22	PMA: 12	PMA: 60
HT29 (colon cancer)	Veh: 43	Veh: 22	Veh: 32
	PMA: 63	PMA: 11	PMA: 23

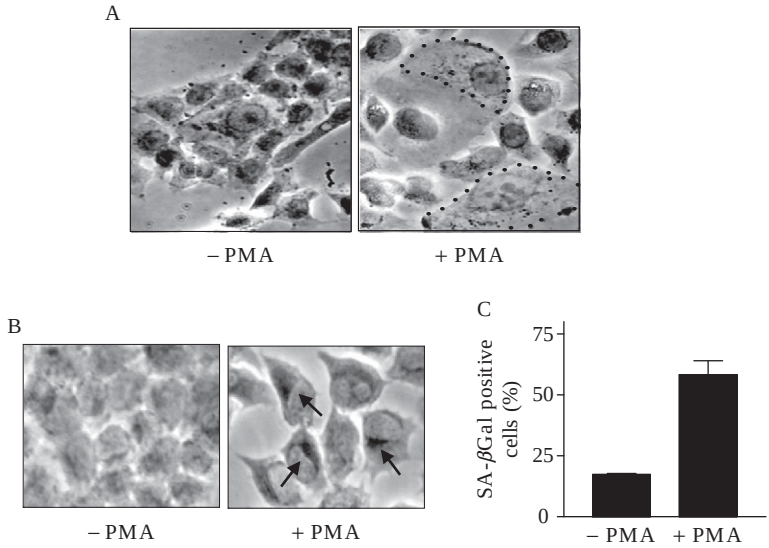

Figure 7.2 **Lung cancer cells undergo senescence in response to PMA. Panel A, morphology of H358 cells treated for 30 min with either vehicle (−PMA) or 100 n*M* PMA (+PMA), assessed 3 days later by light field microscopy. The borders of some cells are highlighted by dotted lines. Panels B and C, H460 cells were treated for 30 min with either vehicle (−PMA) or 100 n*M* PMA (+PMA). Three days later, cells were fixed and stained for the senescence associated-β-galactosidase (SA-β-Gal) marker. Positive (senescent) SA-β-Gal cells in Panel B show typical perinuclear staining (arrows). In Panel C the percentage of senescent cells 3 days after PMA treatment is shown. Results are expressed as mean ± SEM of three independent experiments.**

we strongly recommend testing different brands and lots of serum. The following protocol is used:

1. Plate LNCaP cells at density of 2×10^5 cells per well in a 6-well plate (~70% confluence) in RPMI 1640 supplemented with 10% FBS.
2. Allow cells to attach. Leave cells for 48 h in complete RPMI medium.
3. Prepare a 1 m*M* PMA (LC Laboratories, Woburn, MA) stock solution in either dimethyl sulfoxide (DMSO) or 100% ethanol. Aliquot the solution and keep it at −80 °C. Even after freezing, we have noticed that PMA activity is reduced with time. We recommend preparing a fresh stock every 3 to 4 weeks.
4. Treat cells with PMA for 1 h. Maximum apoptosis is normally observed with 100 n*M* PMA.
5. If inhibitors are used, such as the PKC inhibitor GF109203X (bisindolylmaleimide I), they are normally added 30 min before PMA and left in the medium during the incubation with the phorbol ester.

6. After 1 h incubation with PMA, wash cells twice with prewarmed (37°C) complete medium to remove the PMA. Wash gently to minimize cell detachment.
7. Fresh medium is added and cells are grown for 24 h.
8. Twenty four hours after PMA treatment, the supernatant containing floating cells is collected in a 15-ml conical tube. The plate is washed with 2 ml of PBS and also transferred to the same conical tube.
9. The attached cells are trypsinized and resuspended in 2 ml of PBS, and the cell suspension mixed with that containing the floating cells.
10. Cells are then pelleted at 800 *g* for 5 min at 4 °C. Remove the supernatant and resuspend the cell pellet in 200 μl of PBS.
11. Add 100 μl of the cell suspension on a glass slide (Micro Slides, Single Frosted, Corning; Corning, NY), spread it, and then air-dry. It is possible to let them air-dry for up to 48 h.
12. Fix cells in precooled (−20 °C) 70% ethanol for 20 min.
13. Stain cells with 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI; Sigma, Saint Louis, MO) in PBS for 20 min at 4 °C protected from light. Let the samples air-dry in the dark. The DAPI stock solution is stored at 4 °C in the dark.
14. Finally, mounting medium Fluoromount-G (SouthernBiotech, Birmingham, AL) is added, and the cells are examined by fluorescence microscopy.
15. We normally count 300 cells per preparation and determine the percentage of cells with condensed or fragmented chromatin. PMA 100 n*M* will normally cause ~30% of cells to undergo apoptosis. The percentage of apoptosis normally matches that observed by flow cytometry (sub G0/G1 population).

7. Adenoviral Expression of PKC Isozymes

To express wild-type or dominant-negative PKCs in prostate cancer cells we normally use an adenoviral approach. Although adenoviral delivery of PKCδ is insufficient to trigger an apoptotic response, overexpression of this PKC greatly enhances the PMA effect. Generation of PKC AdVs is described elsewhere (Berkner, 1992; Li *et al.*, 1999). Following is a protocol that we normally use to infect prostate cancer cells, but it is similar for other cancer models that we use in the laboratory, such as lung cancer cells.

1. Plate LNCaP cells at a density of 2×10^5 cell/well in a 6-well plate (~70% confluence) in RPMI 1640 supplemented with 10% FBS (see earlier).
2. After 48 h, infect cells with AdV encoding for a PKC isozyme (Berkner, 1992; Fujii, 2000; Kuroki, 1999; Li, 1999; Ohba 1998) or control LacZ AdV for 14 h at different multiplicities of infection (MOIs) in 2%

FBS-supplemented RPMI 1640 medium. We normally use a MOI of 1 to 300 pfu/cell. For the calculation of the volume of adenoviral stock to be added use the following formula:

$$\text{Volume}(\mu\text{l}) = \frac{\text{MOI(pfu/cell)} \times \text{Number of cells}}{\text{Viral titer(pfu/}\mu\text{1)}}$$

3. After removal of the virus, cells are incubated in RPMI 1640 medium supplemented with 10% FBS for 24 h.
4. Check expression of the corresponding PKC by Western blot. For PKCα we use an antibody from Upstate (Lake Placid, NY) at a 1:1000 dilution. For PKCδ we use an antibody from Cell Signaling (Denvers, MA) at a 1:1000 dilution. Normally, expression of PKCs can be detected 24 h after infection, remaining stable for several days.

8. RNA Interference of PKC Isozymes

To interfere with PKC expression we have used 21 bp dsRNAs from Dharmacon, Inc. (Gonzalez-Guerrico *et al.* 2005). The following target sequences were used: CCATCCGCTCCACACTAA (PKCα), CCATGA GTTTATCGCCACC (PKCδ), GTGGAGACCTCATGTTTCA (PKCε). As a control we used CATCGCTGTAGCATCGTCT. For transfection of siRNAs into LNCaP cells we use either Oligofectamine (Invitrogen) or the A maxa nucleofector at a final concentration of 100 n*M*, although we recommend doing a concentration-response analysis for optimization in each case.

8.1. Transfection with oligofectamine

1. Seed 2×10^5 LNCaP cells/well in a 6-well plate.
2. Change medium to serum-free RPMI 1640 medium for 4 h.
3. Prepare solution #1: For each well, add 200 pmol of the dsRNA (10 μl of a 20 μM solution) to 340μl of Optimen medium for each well.
4. Prepare solution #2 by mixing 12 μl Oligofectamine with 38 μl Optimem for each well.
5. Combine solutions #1 and #2. Mix gently and incubate 15 min at room temperature.
6. While complexes form remove media, and add 1400 μl media/well.
7. Add the mix (400 μl/well) to each well.
8. After 4 h, add 200 μl FBS to each well, the final volume should be 2 ml/well.
9. Check expression of PKCs by Western blot. We have observed maximum depletion at 48 h, lasting up to 96 h in some cases.

8.2. Transfection with the amaxa nucleofector

1. LNCaP cells are seeded 2 to 4 days before transfection, to reach ~60 to 80% confluence at the time of transfection.
2. For transfection of LNCaP cells we use Amaxa cell line nucleofector Kit R, according to the standard protocol provided by the manufacturer. After trypsinization, resuspend cells in 100 μl/reaction of nucleofector Solution R. For 2 plates (6-well each), mix 2×10^6 cells with 240 pmol of the dsRNA (6 μl of a 40 μM solution).
3. Add the cell/siRNA mix to the cuvette. Select and start the T-009 Nucleofector program, according to the manual.
4. Transfer cells from cuvette to complete prewarmed RPMI 1640 medium immediately. Plate cells into the 6-well plates that have been coated with poly-L-lysine from Sigma (St. Louse, MO, see standard protocol).
5. Forty-eight hours later, check expression of PKCs by Western blot.

9. Collection and Storage of CM from LNCaP Cells

CM from PMA-treated prostate cancer cells was shown to have pro-apoptotic activity when added to LNCaP cells (Gonzalez-Guerrico, 2005). Although the nature of the apoptotic factors is not yet fully characterized, it became clear that they act mainly through the activation of the extrinsic apoptotic cascade. For the collection of CM we use the following protocol:

1. Plate LNCaP cells in complete medium in 10-cm dishes, as described previously.
2. Two or 3 days later, when the culture reaches ~70% confluence, treat cells with either 100 nM PMA or vehicle for 1 h.
3. Gently wash the cells twice with prewarmed (37 °C) medium to remove the phorbol ester, trying to minimize cell detachment. Add fresh complete medium.
4. At 24 h, collect CM and pass it through a 13-mm syringe filter (0.45-μm pore size, Fisher Scientific). CM can be dialyzed by use of 12 to 14 kDa cutoff membrane.
5. To assess the apoptogenic activity of the CM, 2 ml/well of the CM are applied to LNCaP cells in 6-well plates, and cells are collected 24 h later for the assessment of apoptosis by DAPI staining.
6. Although we found that CM retained its apoptotic activity up to a week at 4 °C, we strongly recommend the use of fresh CM for each experiment. Freezing and thawing may cause significant loss of apoptotic activity.

10. Determination of Senescence in Response to Phorbol Esters

We found that a short-term treatment with PMA leads to a senescent phenotype in H358 lung carcinoma cells (Oliva *et al.*, 2008). We normally use a combination of flow cytometry, cell counting, [^{3}H] thymydine incorporation, and SA-β-Gal staining for routine assessment of senescence. For this analysis it is important that cells do not reach confluency to avoid inhibition by contact.

10.1. H358 cell culture and PMA treatment

1. Plate H358 lung cancer cells in RPMI 1640 medium supplemented with 10% FBS, penicillin (100 U/ml)-streptomycin (100 μg/ml). Cell number for each type of readout are indicated in Table 7.2.
2. The next day treat cells with PMA (100 n*M*, 30 min).
3. Wash three times with PBS to remove PMA.
4. Add complete medium.

10.2. Flow cytometry

Cells are seeded in duplicates (see Table 7.2) and treated with PMA as described previously. At the desired times (e.g., 24, 48, and 72 h after treatment), process the cells for flow cytometry, as follows:

1. Trypsinize the cells and resuspend them in complete medium.
2. Transfer the cells from each plate to 15-ml conical tubes.
3. Pellet cells by centrifugation at 800*g* (5 min, 4 °C).
4. Discard the supernatant and resuspend the pellet in 500 μl PBS.
5. Add 4.5 ml of 70% ethanol. Allow the cells to fix at 4 °C for at least 30 min before continuing the staining.
6. Collect the cells by centrifugation.
7. Resuspend pellet in 300 to 500 μl of staining solution (0.1 mg/ml propidium iodide, 1 mg/ml RNase A in 0.1% Triton-X 100).

Table 7.2. Cell number for H358 lung cancer cells

Assay	Plate	Cell number	Volume of medium
Flow cytometry	60-mm	400,000 cells/plate	4 ml
Proliferation	12-well plate	100,000 cells/well	1 ml
SA-β-Gal	6-well plate	200,000 cells/well	2 ml

8. Perform the FACS analysis. Samples from different time points should be stored and processed at the same time. Typical results for different cell lines are indicated in Table 7.2.

10.3. [^{3}H]Thymidine incorporation

Seed cells in triplicate wells (see Table 7.2). Treat cells with PMA as described previously. We recommend thymidine incorporation to be determined at various times after PMA treatment (1 to 3 days). Measurements are carried out as follows:

1. Add 3 μCi/ml of [methyl-^{3}H] thymidine (Amersham). Incubate 4 h at 37 °C.
2. Wash cells twice with PBS (2 ml/well).
3. Fix the cells in cold ethanol for 5 min (2 ml/well).
4. Wash twice with PBS.
5. Precipitate the DNA by adding 1 ml/well of ice-cold 20% trichloroacetic acid (TCA, 20 min, 4 °C).
6. Wash with PBS.
7. Add 0.5 *N* NaOH (600 μl/well).
8. Store the plates at −20 °C until all time point samples are ready for measurement.
9. Measure radioactivity in each sample in a scintillation counter (300 μl of sample in 2 ml scintillation fluid/ vial). A 70% inhibition of DNA synthesis is normally observed in H358 cells 24 h after PMA treatment.

10.4. SA-β-Gal staining

Cells are seeded as described in Table 7.2. Treat cells with PMA as described previously. Staining for SA-β-Gal is conducted 3 days later, as follows:

1. Wash cells once with PBS.
2. Fix cells in 2% formaldehyde/0.2% glutaraldehyde in PBS (5 min, room temperature).
3. Wash cell twice with PBS.
4. Incubate cells overnight with 1 ml/well of staining solution: 1 mg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D galactopyranoside), 5 m*M* potassium ferrocyanide, 5 m*M* potassium ferricyanide, 150 m*M* NaCl, and 2 m*M* $MgCl_2$ in 40 m*M* citric acid/sodium phosphate buffer, pH 6.0. The pH conditions are extremely important for development of the reaction. Therefore, a fresh staining solution should be prepared each time, and incubations should be performed at 37 °C in an incubator without CO_2 injector. Stocks solutions of potassium ferrocyanide/ferricyanide (500 m*M* in H_2O) should be aliquoted and stored at −20 °C.
5. Wash cells twice with PBS.

6. Determine the percentage of blue cells by phase contrast microscopy. We normally count ~300 cells/well. Fig. 7.2B shows the typical perinuclear staining (arrows) in positive cells.
7. Plates can be stored in 70% glycerol at 4 °C.

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MOLECULAR CARCINOGENESIS 48:187–195 (2009)

BRIEF COMMUNICATION

PKC-Mediated Secretion of Death Factors in LNCaP Prostate Cancer Cells Is Regulated by Androgens

Liqing Xiao, Anatilde Gonzalez-Guerrico, and Marcelo G. Kazanietz*

Department of Pharmacology and Institute for Translational Medicine and Therapeutics (ITMAT), University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania

Activation of PKCδ in androgen-dependent LNCaP prostate cancer cells leads to apoptosis via the activation of p38 MAPK and JNK cascades. We have recently shown that treatment of LNCaP cells with phorbol 12-myristate 13-acetate (PMA) leads to a PKCδ-mediated autocrine release of death factors, including the cytokines TNFα and TRAIL, and that conditioned medium (CM) collected from PMA-treated LNCaP cells promotes the activation of the extrinsic apoptotic cascade. Interfering with this autocrine loop either at the level of factor release or death receptor activation/signaling markedly impaired the PMA apoptotic response. In the present study we show that this PKCδ-dependent autocrine mechanism is greatly influenced by androgens. Indeed, upon androgen depletion, which down-regulates PKCδ expression, TNFα and TRAIL mRNA induction and release by PMA are significantly diminished, resulting in a reduced apoptogenic activity of the CM and an impaired ability of the CM to activate p38 MAPK and JNK. These effects can be rescued by addition of the synthetic androgen R1881. Furthermore, RNAi depletion of the androgen-receptor (AR) from LNCaP cells equally impaired PMA responses, suggesting that PKC-mediated induction of death factor secretion and apoptosis in LNCaP prostate cancer cells are highly sensitive to hormonal control.

Key words: PKC; PMA; apoptosis; androgens; p38 MAPK; JNK

INTRODUCTION

Apoptosis or programmed cell death is a highly regulated process, in which phosphorylation events play key modulatory roles [1–3]. It is well established that protein kinase C (PKC), a family of serine-threonine kinases, modulates both apoptosis and survival in various cell types [4–7]. There are at least 10 PKC family members classified into classical (α, βI, βII, and γ), novel (δ, ε, η, and θ), and atypical (λ and ζ) isozymes, of which only members of the first two classes are subject to regulation by the lipid second messenger diacylglycerol (DAG). Phorbol esters, widely used PKC activators that mimic the actions of DAG, cause profound effects on cell proliferation and differentiation, and they can also induce survival or apoptotic responses in a strict cell type-dependent manner. The marked heterogeneity observed for phorbol ester responses is a direct consequence of the multiplicity of cellular targets for these compounds, and it is largely determined by the differential pattern of PKC isozyme expression in each cell type as well as the characteristic coupling of individual PKCs to downstream effectors, which include the extracellular-signal regulated kinase (ERK), c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (MAPK), NF-κB, and Stat signaling pathways [4,5,8].

Androgen-dependent prostate cancer cells, such as LNCaP and CWR22-Rv1 cells, undergo apoptosis in response to phorbol 12-myristate 13-acetate (PMA) [9–13] via activation of the novel PKCδ isozyme [12,14]. On the other hand, the novel PKCε isozyme signals for survival in prostate cancer cells [15]. Expression levels of PKC isozymes are deregulated in cancer, including prostate cancer, and disrupting the balance in PKC isozyme expression or function has a considerable impact on cancer progression as well as on the responses to PKC activators [8]. Signaling studies established that PKCδ-dependent apoptosis is mediated by the p38 MAPK and JNK cascades [14,16]. These pathways are well-established mediators of cell death induced by cytokines such as TNFα and tumor

Abbreviation used: PKC, protein kinase C; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; PMA, phorbol 12-myristate 13-acetate; TNF, tumor necrosis factor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; CM, conditioned medium; RNAi, RNA interference; AR, androgen receptor; FBS, fetal bovine serum; ELISA, enzyme-linked immunosorbent assay.

*Correspondence to: Department of Pharmacology, University of Pennsylvania School of Medicine, 1256 Biomedical Research Building II/III, 421 Curie Blvd., Philadelphia, PA 19104-6160.

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necrosis factor-related apoptosis-inducing ligand (TRAIL) [17]. Interestingly, a recent study revealed that PKC activation stimulates the autocrine secretion of death factors from prostate cancer cells, including TNFα and TRAIL. Immunoneutralization of TNFα in conditioned medium (CM) collected from PMA-treated cells significantly impaired its apoptogenic activity. A similar effect is observed upon blockade or RNA interference (RNAi) depletion of TNFα and TRAIL receptors, as well as by interfering with downstream effectors of the extrinsic apoptotic cascade, including the adaptor Fas-associated protein with a death domain (FADD), caspase-8, p38 MAPK and JNK [18], suggesting an essential role for these cytokines in the PKC-mediated autocrine loop.

It has been recently noted that androgens greatly influence the apoptotic responses to phorbol esters in prostate cancer cells. A significant reduction in the ability of PMA to activate apoptotic signaling is observed either upon androgen-depletion of the culture medium or RNAi knock-down of the androgen receptor (AR). A subsequent analysis revealed that androgens modulate the expression of PKCδ in prostate cancer cells at a transcriptional level [19]. We therefore reasoned that PKCδ-dependent induction of the autocrine apoptotic response in prostate cancer cells is modulated by hormonal mechanisms via the AR. To address this issue we decided to analyze whether androgen affects the secretion of death factors from LNCaP cells in response to PKC activation and impact on the activation of pro-apoptotic signaling events. Our results show that phorbol ester-induced autocrine secretion of death factors and cell death are strictly dependent on androgen, suggesting a major role for hormonal control in the modulation of PKCδ-mediated apoptosis in prostate cancer cells.

MATERIALS AND METHODS

Materials

PMA was purchased from LC Laboratories (Woburn, MA). The synthetic androgen methyl trienolone (R1881) was obtained from Perkin-Elmer (Boston, MA). 4′,6-Diamidino-2-phenylindole (DAPI) was purchased from Sigma (St. Louis, MO). Charcoal/dextran-treated fetal bovine serum (FBS) was from Hyclone (Logan, UT). Other cell culture reagents and media were from ATCC (Rockville, MD).

Cell Culture

Human prostate cancer cells LNCaP (passages 2–10), PC3, and DU-145 cells (from ATCC) were cultured in RPMI 1640 medium supplemented with 10% FBS and penicillin (100 U/mL)-streptomycin (100 μg/mL) at 37°C in a humidified 5% CO_2 atmosphere. For steroid depletion, cells were incubated in Phenol red-free RPMI 1640 supplemented with 2% charcoal/dextrane-treated FBS for 48 h, as previously described [19].

Collection of CM

Cells in 10 cm dishes (~70% confluence) were treated with PMA (100 nM) or vehicle (ethanol) for 1 h and then washed twice with medium to remove the phorbol ester or vehicle. After incubation for 24 h, CM was collected, pass through a 0.45 μM filter, and added to fresh LNCaP cells (~70% confluence).

Western Blot Analysis

Western blot was carried out as described [12]. The following first antibodies were used: anti-PKCδ (Transduction Laboratories, Lexington, KY), anti-AR (Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho-JNK, anti-total-JNK, anti-phospho-p38 MAPK, and anti-total-p38 MAPK (Cell Signaling Technology, Beverly, MA). All primary antibodies were used at a 1:1000 dilution. Secondary antibodies conjugated to horseradish peroxidase (Bio-Rad, Hercules, CA) were used at a 1:3000 dilution.

Apoptosis Assays

The incidence of apoptosis was determined by assessing morphological changes in chromatin condensation by fluorescence microscopy after DAPI staining, as described before [12]. The incidence of apoptosis in each preparation was analyzed by counting ~300 cells.

RNA Interference (RNAi)

The following sequences were used: AAGCACUGCUACUCUUCAGCA (AR), and AACAUCGCUGUAGCAUCGUCU (control RNAi). RNAi duplexes from Dharmacon (Lafayette, CO) were transfected with the Amaxa Nucleofector system (Amaxa Biosystems, Gaithersburg, MD) following the instructions provided by the manufacturer. Experiments were performed 48 h after transfection.

RNA Isolation and cDNA Synthesis

RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA). Two micrograms of RNA per sample were reverse transcribed using the First-Strand cDNA Synthesis Kit (Amersham Biosciences, Piscataway, NJ).

Real-Time PCR

PCR primers and fluorogenic probes for human TNFα and TRAIL were purchased from Applied Biosystems (Branchburg, NJ). The probes were 5′end-labeled with 6-carboxyfluorescein (FAM). Each PCR amplification was performed in a total volume of 12.5 μL, containing 6.25 μL of 2× TaqMan Universal PCR Master Mix (Applied Biosystems), commercial target primers (300 nM), the fluorescent probe (200 nM), and 1 μL of cDNA, using an ABI PRISM 7700 Detection System. PCR product forma-

tion was continuously monitored using the Sequence Detection System software version 1.7 (Applied Biosystems). The FAM signal was normalized to endogenous glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Enzyme-Linked Immunosorbent Assay (ELISA)

TNFα and TRAIL levels were determined by ELISA (Pepro Tech, Inc., Rock Hill, NJ), essentially as described previously [18]. Briefly, 100 μL of CM were added into each well and incubated overnight at 4°C. Subsequently, 100 μL of biotin-labeled anti-TNFα or anti-TRAIL antibodies (0.25 μg/mL) were added for 2 h at room temperature. Bound antibodies were detected by incubation with peroxidase-labeled avidine and SureBlue TMB Microwell Peroxidase Substrate from KPL (Gaithersburg, MD), and absorbance was measured at 405 nm. Non-specific binding was blocked with 1% BSA in PBS.

RESULTS

We have previously determined that the apoptotic activity of phorbol esters in LNCaP cells is associated with their ability to promote the release of death factors, which upon binding to death receptors in LNCaP cells trigger the activation of the extrinsic apoptotic cascade. CM collected from LNCaP cells treated with PMA (CM-PMA), but not from vehicle-treated cells (CM-Veh), has apoptogenic activity when added to a fresh culture of LNCaP cells. This activity is essentially undetectable when cells were pretreated with a PKC inhibitor or subject to PKCδ RNAi before the addition of PMA, thus indicating an essential role for the novel PKCδ isozyme in the release of death factors [18]. PKCδ expression is transcriptionally modulated by androgens and androgen depletion reduces PKCδ levels in LNCaP cells, without changes in PKCε or PKCζ levels, and a slight reduction in PKCα levels [19], we reasoned that PMA induction of death factor secretion should be affected when LNCaP cells are cultured in androgen-depleted medium. To address this issue we collected CM-PMA from LNCaP cells growing either in normal medium or in steroid-depleted medium (charcoal-treated) and determined its ability to trigger an apoptotic response in previously untreated LNCaP cells (Figure 1A). Interestingly, the apoptogenic activity of CM-PMA collected from LNCaP cells growing in steroid-depleted medium was markedly reduced compared to that from cells growing in normal medium (Figure 1B). On the other hand, supplementing the steroid-depleted cultured medium with the synthetic androgen R1881 fully restored the apoptotic response of CM-PMA. We have reported that androgen-independent prostate cancer cells also release death factors capable of promoting LNCaP cell apoptosis [18]. However, as expected, the apoptogenic activity of the CM collected in response to PMA from either DU145 or PC3 androgen-independent cells was not affected when these cells were grown in steroid-depleted medium or by addition of R1881 to the steroid-depleted medium (Figure 1C). As previously reported, PKCδ levels are significantly reduced in LNCaP cell subject to steroid depletion and restored by R1881 supplementation (Figure 1D, upper left panel, see also Ref. [20]), but these effects were not observed in DU145 (lower panel) or PC3 cells (upper right panel). Therefore, it is conceivable that the secretion of apoptotic factors from LNCaP cells is regulated by androgen.

Previous analysis of autocrine factors released from LNCaP cells in response to PMA treatment revealed that TNFα and TRAIL, but not FasL, play a significant role in the pro-apoptotic loop [18]. PMA caused a prominent up-regulation in TNFα mRNA levels in LNCaP cells (Figure 2A). Notably, this effect was significantly lower in LNCaP cells growing in steroid-depleted medium. Addition of R1881 to the steroid-depleted medium, which did not significantly affect TNFα levels in the absence of phorbol ester stimulation, restored the ability of PMA to induce TNFα mRNA (levels were indeed doubled compared to those observed in LNCaP cells growing in normal medium, probably reflecting the presence of additional modulatory factors in serum present in normal medium). While the effect of PMA on TRAIL mRNA induction was modest (~2-fold), it was also abolished in steroid-depleted growing cells, and the addition of R1881 fully restored the PMA response (Figure 2B).

PMA promotes a marked release of TNFα from LNCaP cells, as determined by ELISA (Figure 2C and Ref. [18]), an effect that is inhibited by GF109203X (a ''pan'' PKC inhibitor), rottlerin (a PKCδ inhibitor), or PKCδ RNAi [18]. Interestingly, as shown in Figure 2C, the release of TNFα by PMA was blunted in LNCaP cells growing in steroid-depleted medium. This effect was restored by addition of R1881 to the medium. TRAIL levels in CM-PMA were also higher than in CM-Veh, as determined by ELISA (Figure 2D). Moreover, TRAIL levels were significantly lower in CM-PMA collected from LNCaP cells growing in steroid-depleted medium relative to CM-PMA collected from cells in normal medium. As with TNFα, when the steroid-depleted medium was supplemented with R1881, PMA was able to cause a full release of TRAIL.

Next, to further establish the relevance of these findings, we analyzed the effect of AR depletion on the autocrine secretion of death factors. We have previously determined that AR knock-down using RNAi abrogates the apoptotic effect of PMA in LNCaP cells [19]. Delivery of a specific AR RNAi duplex into LNCaP cells caused a significant reduction in AR levels as well as PKCδ down-regulation (Figure 3A, see also Ref. [19]). No appreciable changes in PKCε or PKCζ levels were observed, and a reduction in PKCα was detected (Supplementary Figure 1), consistent

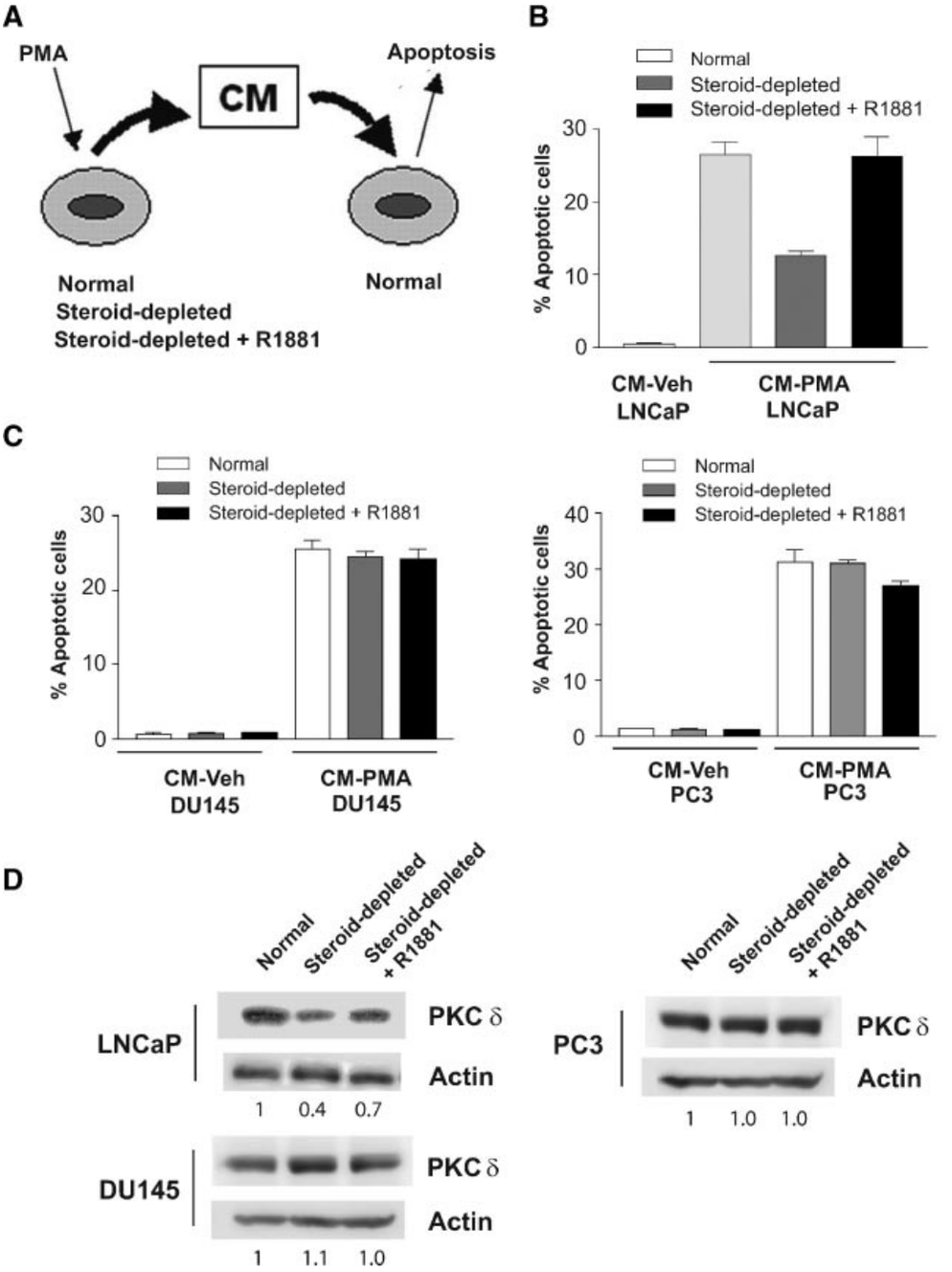

Figure 1. The apoptotic effect of CM from PMA-treated LNCaP cells is androgen-dependent. Panel A: Schematic representation of the experimental approach. Different prostate cancer cells (LNCaP, DU145, or PC3) growing for 48 h in normal medium (RPMI, 10% FBS), steroid-depleted medium (RPMI, 10% charcoal-treated FBS), or steroid-depleted medium supplemented with R1881 (1 nM), were treated with PMA (100 nM, 1 h) or vehicle. After 24 h CM was collected and added to LNCaP cells growing in normal medium. The percentage of apoptotic cells was determined 24 h later by DAPI staining. Panel B: Effect of CM from androgen-dependent LNCaP cells. Panel C: Effect of CM from androgen-independent DU145 and PC3 cells. Panel D: Expression of PKCδ in prostate cancer cells growing in normal medium, charcoal-treated medium, or charcoal-treated medium supplemented with R1881 (1 nM). Expression levels, relative to normal, have been determined by densitometry and are shown below each corresponding Western blot. For apoptosis assays, results were presented as mean ± SD of an experiment performed in triplicate. Two additional experiments gave similar results. *CM-PMA*, conditioned medium from PMA-treated cells; *CM-Veh*, conditioned medium from vehicle-treated cells.

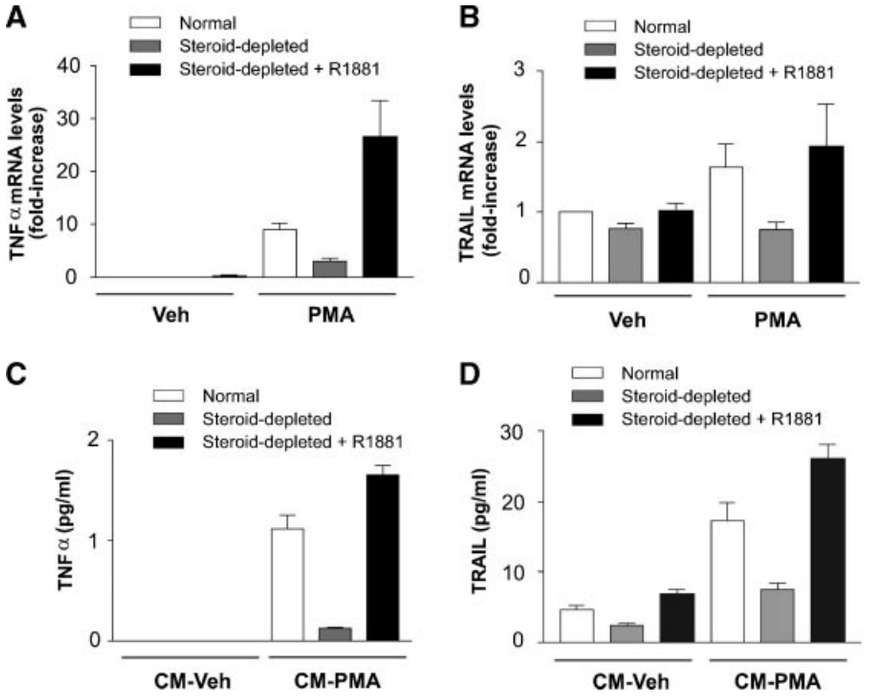

Figure 2. Androgens regulate TNFα and TRAIL mRNA induction by PMA. LNCaP cells were grown in normal medium, steroid-depleted medium, or steroid-depleted medium supplemented with R1881 (1 nM) for 48 h and then treated with either 100 nM PMA (+*PMA*) or vehicle (−*PMA*) for 1 h. Panels A and B: TNFα and TRAIL mRNA levels were determined by real-time PCR 3 h after PMA or vehicle treatment using the TaqMan Gene Expression Assays (Applied Biosystems), and normalized to endogenous GAPDH mRNA levels. Results were expressed as fold-increase relative to mRNA levels in cells growing in normal medium treated with vehicle. Data are presented as mean ± SD of three replicates. Two additional experiments gave similar results. Panels C and D: TNFα and TRAIL levels, as determined by ELISA in CM collected 24 h after PMA or vehicle treatment. Each sample was run by triplicates and results are presented as mean ± SD ($n = 3$). Two additional experiments gave similar results. *CM-PMA*, conditioned medium from PMA-treated cells; *CM-Veh*, conditioned medium from vehicle-treated cells.

with previous results [19]. Interestingly, CM-PMA collected from AR-depleted LNCaP cells had significantly lower apoptotic activity compared to that from cells transfected with a control RNAi duplex (Figure 3B). The partial inhibition may be a consequence of the incomplete AR and PKCδ depletion achieved in these experiments. The ability of PMA to induce TNFα and TRAIL mRNA was markedly diminished in LNCaP cells subject to AR knockdown (Figure 3C and D). Moreover, in AR-depleted LNCaP cells, the release of TNFα and TRAIL by PMA was impaired (Figure 3E and F).

Lastly, since the autocrine-mediated apoptotic effect of phorbol esters in LNCaP cells is mediated by the p38 MAPK and JNK cascades [18], we decided to determine whether androgen depletion impacts on the activation of these signaling pathways. As shown in Figure 4A, addition of CM-PMA to LNCaP cells caused a time-dependent activation of p38 MAPK and JNK. Interestingly, CM-PMA collected from AR-depleted LNCaP cells has a reduced efficacy to activate p38 MAPK and JNK. Likewise, activation of p38 MAPK and JNK by CM-PMA collected from steroid-depleted LNCaP cells was greatly diminished compared to that caused by CM-PMA collected from cells growing in normal medium. This effect was rescued by the addition of R1881 to the medium (Figure 4B). Collectively, our data implicate androgens as key modulators of cytokine release and programmed cell death in response to PKC activation.

DISCUSSION

Apoptosis in response to phorbol esters occurs in a number of cellular models, such as prostate cancer cells, keratinocytes, and hemopoietic cells, and it is mediated in most cases by the novel PKCδ isozyme. Our studies have underscored the existence of a PKC-activated autocrine loop responsible for the apoptosis induced by phorbol esters in prostate cancer cells which involves the death factors TNFα and TRAIL. Constant removal of the factors released to the medium abrogates apoptosis induced by PMA in LNCaP cells, arguing that the autocrine mechanism is necessary for the apoptotic effect [18]. These studies also revealed the involvement of the extrinsic apoptotic cascade, which could be inferred from the induction of caspase-8 cleavage and activation of p38 MAPK, JNK and NF-κB, well-established death receptor effectors. Interfering with the extrinsic apoptotic

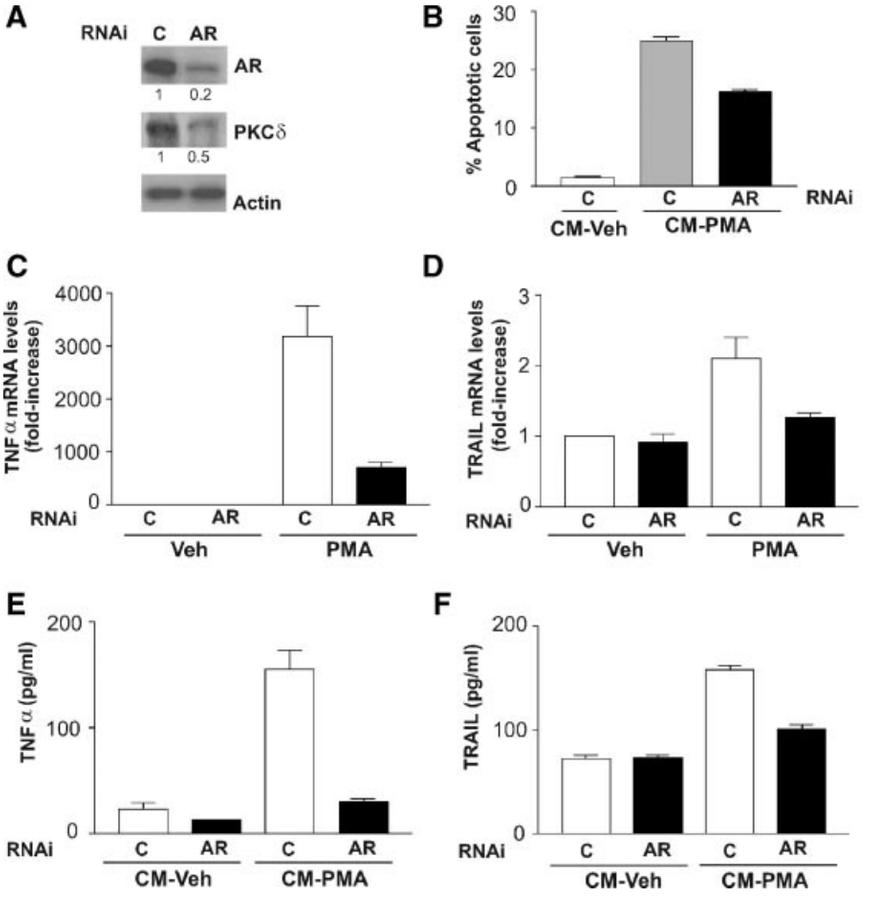

Figure 3. AR RNAi inhibits PMA induction of TNFα and TRAIL. LNCaP cells growing in normal medium were transfected with either an RNAi duplex for AR or a control (*C*) duplex using the Amaxa Nucleofector, and 48 h later treated for 1 h with either 100 nM PMA or vehicle. For mRNA determinations, RNA was extracted 3 h after treatment. For cytokine determinations, CM was collected 24 h after treatment. Panel A: Representative Western blot showing AR depletion and PKCδ down-regulation in AR-depleted cells 48 h after RNAi transfection. Expression levels, relative to control RNAi, have been determined by densitometry and are shown below each corresponding Western blot. Panel B: Apoptotic effect of CM-PMA from LNCaP cells collected from cells subjected to AR or control RNAi. The percentage of apoptotic cells was determined by DAPI staining 24 h after addition of CM-PMA. Panel C: TNFα and TRAIL mRNA levels were determined by real-time PCR 3 h after PMA treatment in LNCaP cells subject to either AR or control RNAi. Results are normalized to endogenous GAPDH mRNA levels and expressed as fold-increase relative to those in control cells treated with vehicle. Panel D: TNFα and TRAIL levels in CM-Veh or CM-PMA collected from LNCaP cells subjected to AR or control RNAi, as determined by ELISA. In all cases, a representative experiment is shown and results are presented as mean ± SD of triplicate samples. Similar results were obtained in two additional experiments. *CM-PMA*, conditioned medium from PMA-treated cells; *CM-Veh*, conditioned medium from vehicle-treated cells.

cascade by various means, including RNAi depletion of caspase-8 or the adaptor FADD, or pharmacological inhibition of p38 MAPK and JNK, reduces the apoptotic effect of PMA [18]. PKCδ has a dual role, both in the secretion of death factors as well as an effector downstream of death receptors [18]. While other studies have established a potential contribution of autocrine factor release to mitogenic and transforming events induced by PKC activation [20–22], our studies established a paradigm of PKC-mediated autocrine apoptotic signaling in prostate cancer cells.

In this manuscript we report that the PKC-triggered pro-apoptotic autocrine loop is sensitive to androgen control. Steroid depletion from LNCaP cell culture medium greatly influenced TNFα and TRAIL mRNA induction and their accumulation in the CM in response to PMA treatment. This is consistent with the reduced apoptogenic activity of PMA in LNCaP cells growing in steroid-depleted (charcoal-treated) serum [18], as well as with the limited ability of CM-PMA from steroid-deprived cells to promote apoptosis. All these effects can be rescued by supplementing the culture medium with R1881, thereby implicating androgens as modulators of the autocrine response. This conclusion is supported by studies using CM-PMA from androgen-independent DU145 and PC-3 cell lines, which were insensitive to androgen depletion. Moreover, the fact that AR RNAi mimics the responses observed

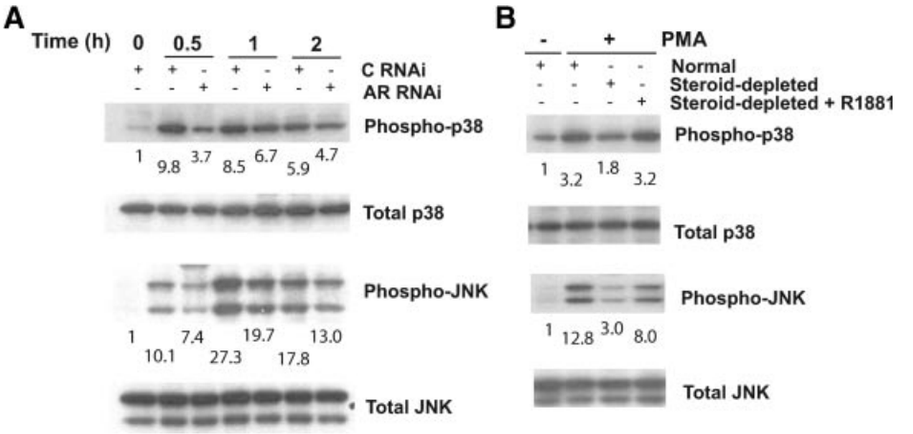

Figure 4. Androgen depletion and AR RNAi impair the ability of CM-PMA to activate p38 MAPK and JNK. Panel A: LNCaP cells growing in normal medium were transfected with RNAi duplexes for either AR or a control (C) duplex using the Amaxa Nucleofector, and 48 h later treated for different times with 100 nM PMA. Panel B: LNCaP cells were grown in normal medium, steroid-depleted medium, or steroid-depleted medium supplemented with R1881 (1 nM) for 48 h, and then treated with either 100 nM PMA (+*PMA*) or vehicle (−*PMA*) for 1 h. For Panels A and B, CM was collected 24 h after treatment and added to LNCaP cells. Cell extracts were prepared and subjected to Western blot analysis using the antibodies indicated in the figures. Phospho-38 and phospho-JNK levels, normalized to the corresponding total levels, were determined by densitometry. Values were expressed as fold-increase relative to $t = 0$ (Panel A) or to cells growing in normal medium and untreated with PMA (Panel B). Values are shown under each corresponding Western blot. Similar results were observed in three independent experiments.

upon androgen-depletion strongly argues for the androgen control of the autocrine release of death factors in response to PKC activation. Only androgens but not other steroids such as glucocorticoids, progesterone, or T3, were able to rescue PMA-induced apoptosis in LNCaP cells growing in steroid-depleted medium, and the androgen rescue was not observed when cells were treated with the AR specific antagonist Casodex [19]. The reduced apoptotic index is independent of the reduced growth rate of steroid-deprived LNCaP cells, as growth factors that reinstate growth of these cells do not restore the apoptotic effect of PMA [19]. Androgen depletion causes a marked reduction in PKCδ expression levels, and since PKCδ mediates the autocrine release of death factors from LNCaP cells it is conceivable that PKCδ down-regulation restricts the release of autocrine factors by PMA. A strict correlation exists between PKCδ levels and the ability of PMA to trigger LNCaP cell death [18,19]. The human PKCδ gene possesses several putative androgen responsive elements (AREs), and at least one located - 4.7 kb from the transcription start site is functionally relevant in vivo in LNCaP cells, as revealed by ChIP analysis and luciferase reporter studies. The transcriptional regulation of PKCδ by androgens in prostate cancer cells has been recently confirmed by others [23]. Androgen-dependent PKCδ up-regulation has been also found in coronary smooth muscle [24]. Interestingly, changes in PKCδ expression have been reported in other models in response to other hormones such as estrogens, vitamins, or mechanical forces [25–28], which like androgens are probably mediated through genomic mechanisms. The ability of androgen to regulate multiple apoptotic proteins [29–31] makes it likely that the impaired apoptotic effect of phorbol esters in androgen-deprived LNCaP cells involves additional mechanisms not necessarily related to PKCδ depletion.

Dissecting the mechanisms that modulate the release and function of cytokines from prostate cancer cells is highly relevant, as therapeutic strategies based on targeting death receptors or their ligands are under development [32–35]. While LNCaP cells have a limited apoptotic response to TNFα and TRAIL when used as single agents, chemoterapeutic drugs or irradiation sensitize cells to these cytokines [36–38]. Anti-androgen therapy represents a standard approach for prostate cancer and it is therefore likely that down-regulation of PKCδ occurs in androgen-responsive tissues upon androgen withdrawal. Not surprisingly, a recent study found reduced PKCδ levels in prostate cancer biopsies from patients undergoing androgen ablation therapy as compared to those from untreated patients [23]. Since PKCδ is not only required for phorbol ester-mediated cytokine release but also acts as an effector downstream of death receptors [18,39], a speculation is that PKCδ down-regulation in androgen-depleted LNCaP cells may also contribute to the resistance to prostate cancer cell killing by cytokines.

In summary, our results revealed that androgens sensitize LNCaP prostate cancer cells to the release of cytokines by phorbol esters. In the absence of androgen, PMA fails to release TNFα and TRAIL, and consequently the overall apoptotic effect of the phorbol ester is impaired. As phorbol ester effects depend on the balance between the activation of pro-

apoptotic and pro-survival PKC isozymes (e.g., PKCδ vs. PKCε), it would be important to determine whether androgen affects the expression of other PKCs. By fine-tuning the expression of PKC isozymes, androgens may greatly alter the effectiveness of chemotherapeutic agents that depend on PKC activation. Of interest, studies have shown that the cell killing effects of etoposide and radiation are dependent on PKCδ [4,40,41]. Given the emerging interest in PKCδ as a therapeutic target [5,8,41–43], and considering that PKC activators are in clinical trials for various types of cancers and greatly enhance the antitumor effects of other agents and radiation in prostate tumors [15,44–46], our studies suggest that modifiers of PKCδ expression such as hormonal control may have significant impact on the responsiveness to therapeutic agents.

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